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A new set of small, extrachromosomal expression vectors for *Dictyostelium discoideum*

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ABSTRACT

A new set of extrachromosomal *Dictyostelium* expression vectors is presented that can be modified according to the experimental needs with minimal cloning efforts. To achieve this, the vector consists of four functional regions that are separated by unique restriction sites, (1) an *Escherichia coli* replication region, and regions for (2) replication, (3) selection and (4) protein expression in *Dictyostelium*. Each region was trimmed down to its smallest possible size. A basic expression vector can be constructed from these modules with a size of only 6.8 kb. By exchanging modules, a large number of vectors with different properties can be constructed. The resulting set of vectors allows most basic expression needs, such as immuno blotting, protein purification, visualization of protein localization and identification of protein–protein interactions. In addition, two genes can be simultaneously expressed on one vector, which yields far more synchronous levels of expression than when expressing two genes on separate plasmids.

1. Introduction

Gene expression is an important element in the research of protein function. In contrast to mammalian systems, no commercial expression vectors are available for the model organism *Dictyostelium dicoideum* and useful components for gene expression such as resistance cassettes and fusion tags have been adapted for use in *Dictyostelium* on a need-to-use basis by different laboratories. As a result, there is now a wide variety of vectors available for diverse expression needs. For visualization of proteins there are several options that allow fusion to green fluorescent protein (GFP) or red fluorescent protein (mRFPmars) (Fischer et al., 2004; Levi et al., 2000). There is also a large number of vectors available for protein purification and epitope tagging (Knetsch et al., 2002; Manstein et al., 1995) and for tandem affinity purification (TAP) tagging (Koch et al., 2006; Meima et al., 2007; Puig et al., 2001) to identify protein–protein interactions. In addition, a small number of vectors has been adapted for use with the Gateway system, which allows genes to be cloned using specific recombinase enzymes (Thomason et al., 2006).

Despite of the impressive amount of vectors that the community has constructed and made available, the large heterogeneity of the different vectors poses some practical problems. A gene that has been prepared for fusion to a tag in one vector is often not compatible for expression in another vector. This necessitates either a PCR amplification of the gene with compatible restriction sites or the introduction of a double stranded linker as an adapter between the gene and the fusion tag. Furthermore, the properties of the different vectors often show a trade-off between ease of construction and ease of use in *Dictyostelium*. Integrating vectors are small and cloning of expression constructs is relatively easy, but transfection efficiency in *Dictyostelium* is low and it can take up to several weeks to obtain the desired clones. Extrachromosomal vectors on the other hand have high transfection efficiency and have no need for clonal selection. However, extrachromosomal vectors based

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on the endogenous *Dictyostelium* plasmid Ddp1 are often substantially larger than integrating vectors and are therefore more difficult to clone, whereas those based on Ddp2 require to be co-transfected with a second vector in order to maintain extrachromosomal replication.

To facilitate the investigation of gene function in *Dictyostelium* we have designed a new set of expression vectors that has very favorable properties for both expression in *Dictyostelium* and for ease of cloning in *E. coli*. The *ab initio* design has two main features, (1) reduce the size as much as possible to allow large genes to be cloned without difficulties and (2) make the plasmid modular to enable it to be modified according to experimental needs. By using a consistent distance of the constructed fusion tags to the multiple cloning site (MCS), a single gene of interest can be fused in frame to a variety of different tags. The resulting set of vectors allows most basic expression needs, such as immuno blotting (FLAG-epitope), protein purification (Glutathione-S-transferase (GST)), localization (GFP and mRFPmars) and protein–protein interaction (TAP) with minimal cloning efforts. Finally, the design of the vector allows further modifications to be made, so that the functions of the vector can easily be expanded for future needs.

2. Materials and methods

2.1. Culture conditions and transformation of Dictyostelium cells

Dictyostelium AX3 cells were used for all experiments. Cells were cultivated on 9 cm Petri dishes containing 10 ml of HG5 medium. For transfection, 15 μl miniprep DNA (approximately 2 μg) was electroporated as described (Howard et al., 1988). The used field strength was 2 kV/cm, capacitance was 50 μF and a 13 Ω resistance was placed in series. Selection marker was added to the cells at 5–18 h after electroporation. Final concentration of blasticidin and G418 was 10 μg/ml. Selection with hygromycin was done at 50 μg/ml. Visible colonies appeared about 3 days after addition of the selection marker.

2.2. DNA cloning

All vectors were constructed using standard cloning methods. DNA minipreps were performed using the alkaline lysis protocol as described in (Sambrook et al., 1989). Restriction enzymes were obtained from Fermentas, New England Biolabs and Roche. PCR amplifications were done using Phusion DNA polymerase (Finnzymes). After PCR, the sequence of all open reading frames was verified by DNA sequencing. Recombinant DNA was transformed using the calcium chloride method (Inoue et al., 1990). *E. coli* XL10-Gold cells (Stratagene) were used for regular cloning and DB3.1 cells were used for gateway cloning.

2.2.1. Expression cassette

The expression cassette was created as follows. The *act15* promoter fragment (abbreviated as A15P in the figures) was obtained from plasmid MB74 (Veltman and Van Haastert, 2006), with the following modification. The XbaI site on the 5′ end of the promoter fragment was converted to an XhoI site by inserting the dimerized oligonucleotide 5′-cta gtc tcg aga-3′. The SpeI–HindIII flanked *act8* terminator (abbreviated as A8T in the figures) was obtained by PCR on plasmid MB74 using primers 5′-gca gat cta gta cta gtt aaa taa ata aat tat tta ata aat aat a-3′ and 5′-tcc aag ctt tat ctt ttt g-3′. Templates for the PCR amplification of the fusion tags are listed in Table 1. Two BglII sites were present within the TAP tag sequences (Puig et al., 2001) and were removed using the QuickChange method (Stratagene) (both silent mutations).

2.2.2. E. coli and Dictyostelium replication region

The HindIII–NgoMIV flanked *Dictyostelium* replication cassette was taken as a restriction fragment from MB12n (Heikoop et al., 1998b). Both of the HindIII sites present within the Ddp1 sequence were removed by site directed mutagenesis. The NgoMIV–BamHI flanked *E. coli* replication cassette was obtained by PCR on plasmid pBluescript SK− (Stratagene) using primers 5′-cgc tcg agg ccg gca gag gcg gtt tgc gta-3′ and 5′-ggg agc tcg gat ccc gct aca ggg cgc gtc ag-3′.

2.2.3. Resistance marker

The XhoI–BglII flanked *act6* promoter fragment (abbreviated as A6P in the figures) that drives the expression of the resistance genes was amplified from vector MB12n using primers 5′-gcg ctc gag ttt ttt aaa taa aaa atg gg-3′ and 5′-aga tct gcg ttt ata tta tat tta ttt a-3′. The XbaI–BamHI flanked *mhcA* terminator (abbreviated as MyoT in the figures) was amplified from vector pBIG-GFP-myo (Moores et al., 1996) using primers 5′-gcg tct aga atc aat ttg att tct tct t-3′ and 5′-gcg gga tcc att tta ttt aat ata cta a-3′. Promoter and terminator fragment were ligated adjacent to each other in pBluescript SK−, resulting in plasmid pDM261.

The genes that confer resistance to G418, hygromycin and blasticidin are from bacterial origin and have previously been adapted for use in *Dictyostelium*. The adapted genes have some minor modifications on their 5′ and 3′ ends. Although these modifications are not necessarily essential for proper function in *Dictyostelium*, we preserved these modifications and used the *Dictyostelium* adapted sequences instead of the original bacterial sequences as templates for PCR. The neomycin phosphotransferase gene from the Tn5 transposon (Beck et al., 1982) was adapted for expression in *Dictyostelium* in vector pB10S (Nellen and Firtel, 1985) and is also present in vector MB12neo (Heikoop et al., 1998b). The gene was amplified from MB12neo using primers 5′-gcg gat cca aaa tgg atg gtg aag atg-3′ and 5′-gca cta gtt cag aag aac tcg tca ag-3′. The blasticidin deaminase gene from *Bacillus cereus* (Itaya et al., 1990) was adapted for use in *Dictyostelium* in plasmid pBsr2 (Sutoh, 1993) and is also present in vector MB12n (Heikoop et al., 1998b). The gene was amplified from MB12n using primers 5′-gcg gat cca aaa tgg atc aat tta ac-3′ and 5′-gca cta gtt taa ttt cgg gta ta-3′. Finally, the hygromycin phosphotransferase gene from *Escherichia coli* (Gritz and Davies, 1983) was adapted for use in *Dictyostelium* in the vector pHygTm(plus)/pG7 (a kind gift from Dr. Jeff Williams). The adapted gene was amplified from this

Table 1
Origin of the fusion tags that are used in the modular expression vector.

Name	Description and source
GFP	Green fluorescent protein (S65T) from Clontech plasmid pS65T-C1 (GenBank acc. no. U36202)
mRFPmars	Monomeric red fluorescent protein from plasmid 339-3, which sequence is identical to GenBank acc. no. AY679163, but without the linker sequence and 6xHis tag
GST	Glutathione-S-transferase followed by a thrombin cleavage site, from plasmid pDXA-GST (GenBank acc. no. AJ510166)
TAP	Two IgG binding domains of *Staphylococcus aureus* protein A and a calmodulin binding peptide separated by a TEV protease cleavage site. Taken from plasmid pBS1761 (N-terminal tag) and pBS1479 (C-terminal tag), described in (Puig et al., 2001)
FLAG	FLAG epitope tag encoding the amino acid sequence DYKDDDDK

vector using primers 5′-gcg gat cca aaa tgg atc aat tta ac-3′ and 5′-gca cta gtt tag tta gcc tcc-3′.

The BamHI–SpeI flanked resistance genes were ligated in vector pDM261 that was digested with BglII and XbaI, placing them in between the *act6* promoter and *mhcA* terminator.

2.2.4. Shuttle vector

Shuttle vector pDM344 was created as follows. The expression cassette was amplified from pDM304 using primers 5′-ggc cgg cta aaa aaa att ttt at-3′ and 5′-ggc cgg cta tct ttt tga ttt tc-3′ and ligated into pBluescript SK− digested with EcoRV. The expression cassette was subsequently excised from this vector with HindIII/EcoRI and ligated into pUC18 digested with HindIII/EcoRI, resulting in vector pDM344.

2.3. Fluorescence microscopy

Fluorescence was observed on a Zeiss LSM 510 confocal laser scanning microscope equipped with a Zeiss plan-apochromatic 63× numerical aperture 1.4 objective. For excitation of GFP and mRFPmars a 488 nm argon/krypton laser and a 543 helium laser were used, respectively. Fluorescent light was filtered through a BP500-530 (GFP) or IR LP560 (mRFPmars) filter and detected by a photo multiplier tube.

3. Results and discussion

A new *Dictyostelium* expression vector was designed that was both modular and as small as possible. To achieve the modularity, four functional regions were first identified, (1) an expression region, (2) an *E. coli* plasmid replication region, (3) a *Dictyostelium* plasmid replication region and (4) a resistance marker region (Fig. 1). A cassette was constructed for each functional region. All cassettes were trimmed down to their smallest possible size and flanked by the indicated unique restriction sites. If necessary, these sites were made unique by site directed mutagenesis.

3.1. Dictyostelium replication cassette

Two types of extrachromosomal vectors are currently available for *Dictyostelium*, those based on Ddp1 and those based on Ddp2 (Ahern et al., 1988; Knetsch et al., 2002). The Ddp2-based expression vectors are generally smaller, as the REP gene that is needed for extrachromosomal replication is placed on a second plasmid that is co-transfected with the expression vector. Despite the substantially larger size, we decided to use Ddp1 as a backbone for extrachromosomal replication as this circumvents the need for co-transfection and reduces the cell-to-cell variation of expression (Levi et al., 2000).

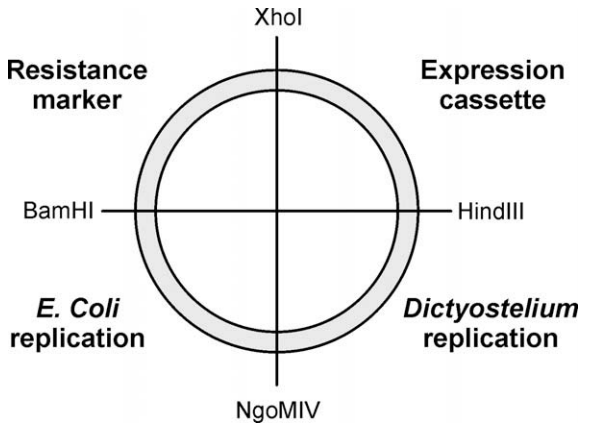

Fig. 1. Design of the modular vector. The expression vector consists of four different modules that are separated by unique restriction sites and assembled according to indicated topology.

Ddp1 contains several putative genes, named G1 to G5 for genes transcribed during vegetative growth and D1–D6 for genes transcribed during development (Gurniak et al., 1990; Kiyosawa et al., 1994; Noegel et al., 1985). A minimal fragment containing only the origin of replication and the G5 gene can still support extrachromosomal replication (Kiyosawa et al., 1995), but plasmids containing this minimal fragment are rapidly lost from the population when selective pressure is removed, indicating that replication and partitioning is somewhat impaired. For our purposes, this minimal fragment can be used as a *Dictyostelium* replication region, but the observed improper replication can lead to practical problems. Plasmid loss leads to cell death when cells are maintained under selective pressure and reduces the net growth rate of the population. A possible integration event of the plasmid into the genome would lead to a selective growth advantage in this case, potentially leading to changes in phenotype and thus irreproducible results. It is therefore preferable to use a Ddp1 fragment that better supports plasmid replication.

Vector MB12n contains the G4/D5 gene and a fragment of the D1 gene in addition to the origin of replication and the G5 gene (Fig. 2A) (Heikoop et al., 1998a). This Ddp1 region is similar to that used in other extrachromosomal vec-

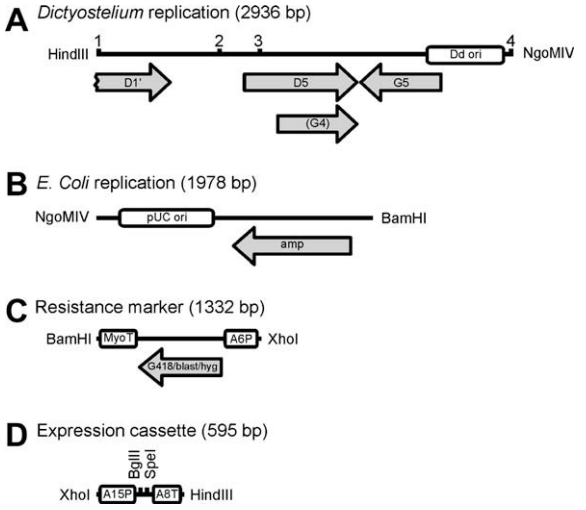

Fig. 2. Schematic overview of the composition of the four modules. All drawings are on the same scale. A15P and A6P are the *act15* and *act6* promoter, respectively. A8T and MyoT are the *act8* and *mhcA* terminator, respectively. The printed size of the resistance marker is that of the cassette containing the neomycin phosphotransferase gene.

tors such as pATANB43, pLittle and pJK1 (Dynes and Firtel, 1989; Levi et al., 2000; Pitt et al., 1992). The additional sequences promote proper plasmid replication and cells carrying these vectors have a relatively normal growth rate when cultivated under selective pressure. To investigate how much the size of the Ddp1 fragment reduced without affecting growth rate, a truncation series was made, incrementally removing the partial D1 gene, the start of the D5 gene and the start of the putative G4 gene, indicated by point 2, 3 and 4, respectively in Fig. 2A. Cells were transfected with plasmids carrying these truncated sequences and the transformation efficiency and growth rate of the cells under selective pressure was determined.

The partial D1 gene is unlikely to lead to the expression of functional protein and may therefore be dispensable for plasmid replication. Somewhat surprisingly, cells carrying plasmids in which the partial D1 gene was removed showed an increased doubling time from about 12 h to 20 h when cultivated under selective pressure. To investigate why the partial D1 gene is required, we co-transfected this short G418 resistant vector with a hygromycin resistant vector containing the longer (regions 1–5) Ddp1 fragment. We observed a similar reduction in growth rate when maintaining these transfected cells under G418 + hygromycin selective pressure, suggesting that the D1 region most likely is needed in cis, and not as a possible protein product. It should be noted that growth rate of the cells returned to normal when G418 selective pressure was removed.

Further trimming of the Ddp1 fragment by removal of the start of the D5 gene and the start of the putative G4 gene incrementally increased the doubling time under selective pressure up to 3 days and 5 days, respectively. After these findings, the full-length Ddp1 fragment as used in vector MB12n and indicated in Fig. 2A, was used as the *Dictyostelium* replication region for the modular vector.

Table 2
List of vectors that have been submitted to the *Dictyostelium* Stock Center. (A) Individual tags that can be ligated into the expression cassette. (B) Complete expression vectors. (C) Shuttle vector for expression of a second gene. A minimal set from which all other listed vectors can be constructed is listed in bold. Annotated sequences of this minimal set have been submitted to GenBank.

A

Plasmid name	Tag	GenBank acc. no.
1.21	N-terminal GFP	EU912543
pDM131	N-terminal mRFPmars	EU912545
pDM193	N-terminal GST	EU912549
pDM229	N-terminal TAP	EU912547
pDM276	C-terminal TAP	EU912548
pDM313	C-terminal GFP	EU912544
pDM312	C-terminal mRFPmars	EU912546
pDM347	Gateway conversion cassette	EU912550

B

Plasmid name	N-terminal tag	C-terminal tag	MCS	Resistance	GenBank acc. no.
pDM304			BglII/SpeI	G418	EU912539
pDM326			BglII/SpeI	Blasticidin	EU912541
pDM358			BglII/SpeI	Hygromycin	EU912542
pDM317	GFP		BglII/SpeI	G418	
pDM318	mRFPmars		BglII/SpeI	G418	
pDM314	GST		BglII/SpeI	G418	
pDM320	FLAG		BglII/SpeI	G418	EU912540
pDM315	TAP		BglII/SpeI	G418	
pDM323		GFP	BglII/SpeI	G418	
pDM324		mRFPmars	BglII/SpeI	G418	
pDM321		TAP	BglII/SpeI	G418	
pDM351	GFP		Gateway	G418	
pDM352	mRFPmars		Gateway	G418	
pDM353		GFP	Gateway	G418	
pDM354		mRFPmars	Gateway	G418	
pDM448	GFP		Gateway	Hygromycin	
pDM449	mRFPmars		Gateway	Hygromycin	
pDM450		GFP	Gateway	Hygromycin	
pDM451		mRFPmars	Gateway	Hygromycin	

C

Plasmid name	N-terminal tag	C-terminal tag	MCS	GenBank acc. no.
pDM344			BglII/SpeI	FJ402941
pDM327	GFP		BglII/SpeI	
pDM328	mRFPmars		BglII/SpeI	
pDM329		GFP	BglII/SpeI	
pDM330		mRFPmars	BglII/SpeI	
pDM410	GFP		Gateway	
pDM411	mRFPmars		Gateway	
pDM412		GFP	Gateway	
pDM413		mRFPmars	Gateway	
pDM414			Gateway	

3.2. E. coli replication cassette

Replication of *E. coli* plasmids has been extensively studied and the functional regions responsible for replication have accurately been described, facilitating the determination of suitable boundaries for a minimal *E. coli* replication cassette. A 2 kb region of plasmid pBluescript II SK(−) that contained both the ampicillin resistance gene and the pUC origin of replication was amplified using PCR (Fig. 2B). Minipreps of cells containing this smaller pBluescript II SK(−) fragment yielded similar amounts of DNA as those containing full-length pBluescript II SK(−) and transformation efficiency in *E. coli* was undiminished (data not shown).

3.3. Dictyostelium resistance marker cassette

The antibiotics blasticidin, G418 and hygromycin were used as selection markers for the modular expression vector. The resistance genes for these selection markers can hardly be made any smaller, but the promoter and terminator regions that drive their expression in many current *Dictyostelium* vectors are often unnecessarily large and possibly can be trimmed. The promoter of the *act6* gene gives rise to high and constitutive levels of expression and is used to drive expression in many *Dictyostelium* vectors (McKeown and Firtel, 1981). An approximately 720 bp fragment of the *act6* promoter was successfully used in the first *Dictyostelium* G418 resistance cassette of vector pB10

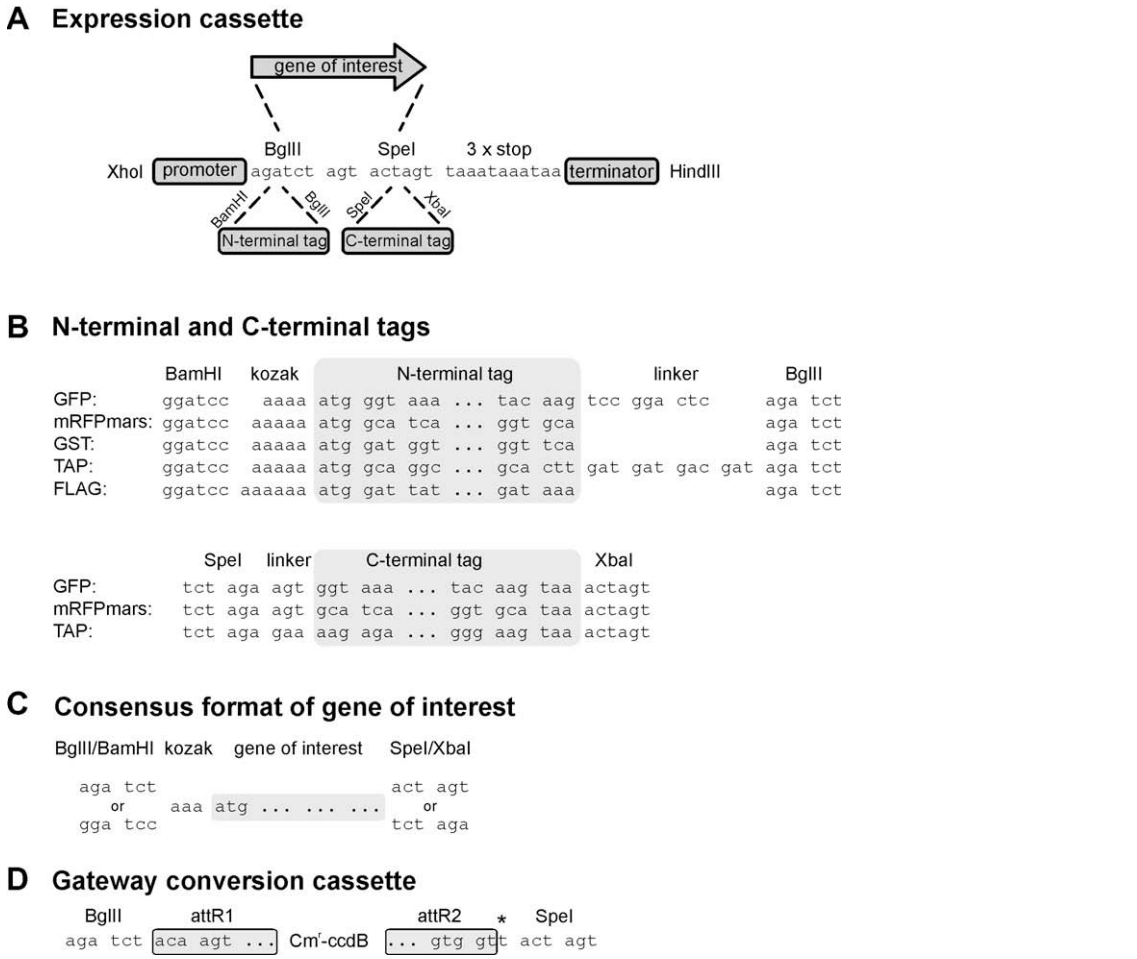

Fig. 3. Details of the expression cassette. (A) Sequence of the multiple cloning site (MCS). The gene of interest is cloned in between the BglII and SpeI site. N-terminal tags are inserted upstream of the MCS by ligating them as BamHI/BglII fragments into the BglII site. In a similar fashion, C-terminal tags are inserted as SpeI/XbaI fragments into the SpeI site, which places them downstream of the MCS. (B) Sequence of the N-terminal and C-terminal tags before insertion into the MCS. All N-terminal tags contain a kozak sequence and a start codon to initialize translation. C-terminal tags contain a stop codon, but do not have a kozak sequence or start codon. Please note that although the size of the linkers varies for the different tags, the reading frame of all tags relative to the restriction sites of the MCS is consistent. (C) Design of a gene of interest that fuses in frame to each N-terminal and C-terminal tag. The 5′ restriction site can be either BglII or BamHI. The open reading frame of the gene is marked by the grey box. The 3′ restriction site can be either SpeI or XbaI. All genes must contain their own kozak sequence and start codon to initialize translation when no N-terminal tag is present. The distance between the start codon and the BglII (or BamHI) site must be a multiple of three for correct N-terminal fusion, thus the kozak sequence can be either 3× a (shown) or 6× a. For a gene to fuse in frame to C-terminal tags, the base prior to the SpeI (or XbaI) site must be the last base of a codon. (D) Sequence of the Gateway conversion cassette. The attR recombination sequences are marked by the grey box. To ensure that Gateway entry clones to fuse in frame to the C-terminal tags of the modular expression vector, a single base was inserted downstream of the attR2 sequence (marked by *).

(Nellen and Firtel, 1985; Nellen et al., 1984). Later work revealed that a smaller 220 bp *act6* promoter fragment resulted in similar levels of expression (Hori and Firtel, 1994). We selected this smaller *act6* promoter fragment to drive the expression of the resistance genes. The first 256 bp directly downstream of stop codon of the *Dictyostelium mhcA* gene were selected as a terminator for the resistance genes. A consensus polyadenylation signal is found 69 bp downstream of the *mhcA* stop codon. Inspection of expressed sequence tags of *mhcA* (www.dictybase.org) reveals that the cleavage site for the *mhcA* transcript is expected about 100 bp downstream of the stop codon, indicating that the selected fragment is sufficient for cleavage and polyadenylation of the nascent transcript.

Vectors carrying the G418, hygromycin and blasticidin resistance genes under control of the *act6* promoter and *mhcA* terminator (Fig. 2C) were electroporated to *Dictyostelium* and transfectants were selected at 10 μg/ml G418, 50 μg/ml hygromycin or 10 μg/ml blasticidin, respectively. Over 1000 colonies were visible about 3 days after transfection in all three cases. Doubling time of blasticidin resistant cells on Petri dish was 12 h, which is similar to that of wild type cells. G418 and hygromycin resistant cells grew somewhat slower with a doubling time of 15 h. However, a 5-fold increase of the concentration of the selection markers had little further effect on the growth rate, indicating that resistance cassettes grant robust resistance to their respective selection markers.

3.4. *Dictyostelium* expression cassette

A gene of interest can be cloned in the modular vector between the unique BglII and SpeI sites, which places it in between an *act15* promoter and *act8* terminator (Fig. 2D). Alternatively, BamHI or XbaI can be used to insert a gene, as these enzymes are compatible with BglII and SpeI, respectively.

To allow detection on western blots or visualization in fluorescent microscopy a gene of interest often needs to be fused to an N- or C-terminal tag. A number of commonly used tags was created for these purpose (Tables 1 and 2A). N-terminal tags can be inserted as a BamHI/BglII fragment into the BglII site of the expression cassette, which places them upstream of the MCS and recreates the unique BglII site (Fig. 3A). All of the N-terminal tags contain a kozak sequence and a start codon to initialize translation. Most importantly, the reading frame of all tags in relation to the BglII and SpeI sites is identical. As a consequence, a gene of interest that is designed to fuse in frame with one N-terminal tag, will fuse in frame to all N-terminal tags. In a similar fashion, a number of C-terminal tags is available that can be inserted in the SpeI site of the expression cassette. The start codons of these tags have been removed, preventing their expression when no gene is fused in front of it. The reading frames of the C-terminal tags are also consistent with each other (Fig. 3B) and a gene that will fuse in frame to one C-terminal tag will fuse in frame to all other C-terminal tags. Proper function of vectors with GFP, mRFPmars, GST and FLAG tags was confirmed (not shown). The sequence of vectors with a TAP tag has been verified, but these vectors have not yet been used in experiments.

The reading frame that is required for a gene of interest in order to be compatible with the N- and C-terminal tags is shown in Fig. 3C. For correct fusion to N-terminal tags, the first base after the BglII site needs to be the first base of a codon of the gene of interest. For proper C-terminal fusion, the last base before the SpeI site needs to be the last base of a codon of the gene of interest.

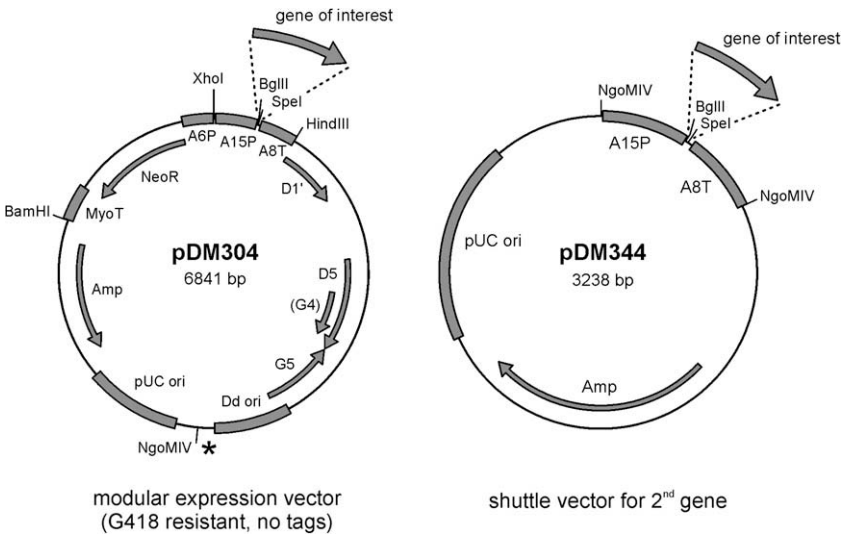

Fig. 4. Overview of two of the designed vectors. pDM304, a G418 resistant expression vector without any fusion tags is shown on the left. The shuttle vector that is used to express a second gene on the modular expression vector is shown on the right. After insertion of a gene of interest, the expression cassette can be excised from the shuttle vector using NgoMIV and ligated in the NgoMIV site of the modular expression vector (indicated by a *).

The Gateway system (www.invitrogen.com) has been presented as an alternative way to clone genes and a large number of destination vectors for expression in bacterial, mammalian, insect and yeast cells are commercially available. A limited number of destination vectors has also been constructed for expression in *Dictyostelium* (Thomason et al., 2006). The biggest advantage of the Gateway system is that all available expression vectors use a consistent reading frame and a compatible gene fragment can thus be expressed in any of these vectors. To enable the expression of genes that were designed for the Gateway system in the modular vectors, a Gateway conversion cassette was created (Fig. 3D). Any vector that is constructed with the here presented modules can be converted into a Gateway destination vector by inserting the conversion cassette as a BglII/SpeI fragment into the BglII/SpeI site of the MCS. The reading frame of the conversion cassette was constructed such that Gateway entry clones fuse properly in frame to either N-terminal or C-terminal tag.

A large number of expression vectors can be created by using different combinations of modules. Some of these

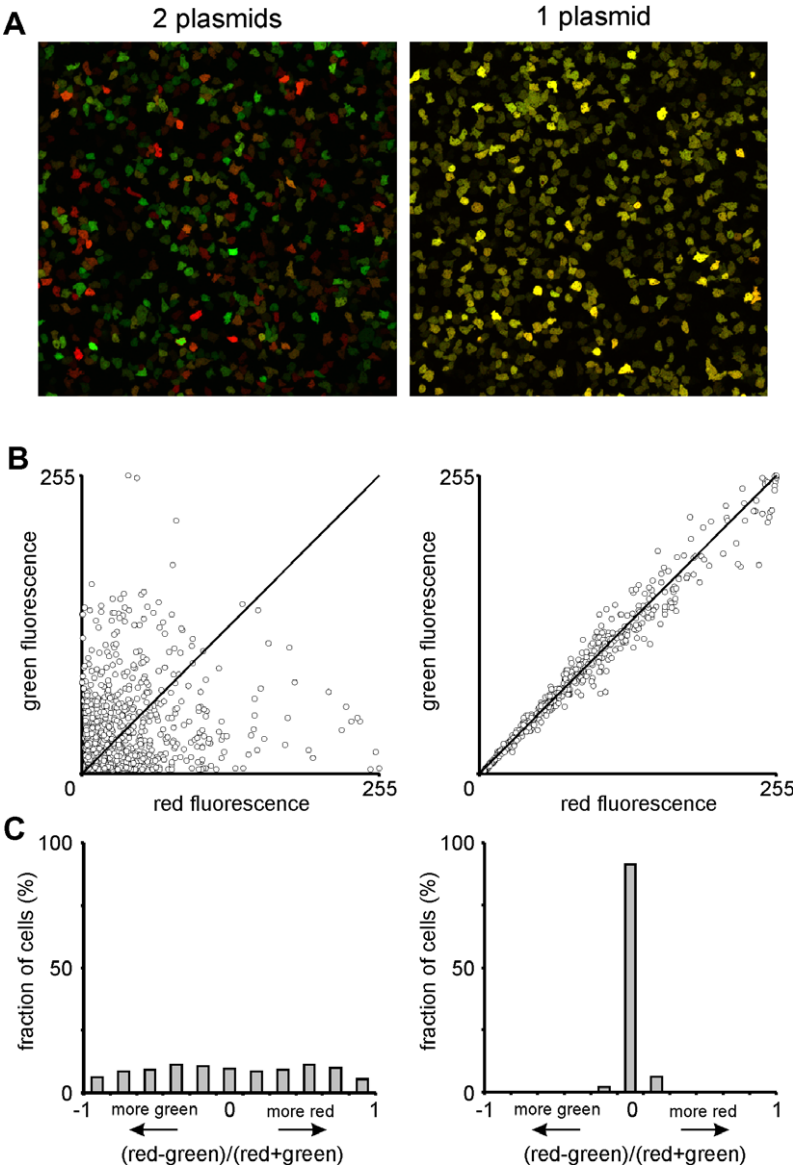

Fig. 5. Cell-to-cell variation of expression of GFP and mRFPmars when both are expressed on different vectors or on the same vector. (A) Image of cells expressing GFP and mRFPmars on two different plasmids (left panels) and on one plasmid (right panels). (B) The amount of red and green fluorescence of each cell was measured and plotted as a dot, where the x-coordinate is the amount of red fluorescence and the y-coordinate is the amount of green fluorescence. The plotted line is that of the function $y(x) = x$. (C) The relative amount of green and red fluorescence of each cell was calculated as (red − green)/(red + green), which yields a number between −1 (cells are completely green) and 1 (cells are completely red). Resulting values were plotted as a histogram.

vectors have already been constructed during this study and have been made available through the *Dictyostelium* Stock Center (Table 2 and www.dictybase.org). A minimal subset of vectors that contains each functional module at least once is marked in bold in Table 2. All vector combinations can be constructed from this minimal set and sequences of these plasmids have been made available through GenBank (http://www.ncbi.nlm.nih.gov/genbank/). Final expression vectors are only 7 kb in size, which is more than 3 kb smaller than previously described Ddp1-based extrachromosomal vectors (Levi et al., 2000; Pitt, 1993). The relatively small vector size allowed the routine cloning of genes of up to 7 kb. Larger genes, resulting in vector sizes of over 18 kb, could also be constructed, but correct clones were obtained at a lower frequency in this case.

3.5. Co-expression

Co-localization experiments require the expression of two fluorescently tagged proteins in one cell. The conventional approach to achieve this is to clone each gene on a vector with a different selection marker. An alternative way would be to express both genes on a single vector. This saves the use of a selection marker and thus allows co-expression of two genes in a double-knockout background where blasticidin and hygromycin resistance markers have already been integrated into the genome. To enable the expression of two genes on one vector, a shuttle vector was constructed that contains an exact copy of the expression cassette in between two NgoMIV sites (Fig. 4). The expression cassette itself is unchanged and all operations that can be performed on the expression cassette of the modular expression vector can also be performed on the expression cassette of the shuttle vector, such as the insertion of tags or the conversion to a Gateway destination vector. After a gene of interest has been inserted into the shuttle vector, the entire cassette can be excised using NgoMIV and ligated into the unique NgoMIV site of the modular expression vector. The recognition sequence of NgoMIV is gccggc, which is extremely rare in *Dictyostelium* genes and most genes will therefore be suitable for double expression without the need to knock out endogenous NgoMIV sites.

To characterize the properties of a vector that simultaneously expresses two genes, we constructed a vector that expressed both GFP and mRFPmars by ligating the NgoMIV fragment of pDM327 into the NgoMIV site of pDM318 (see Table 2). The presence of two identical promoter and terminator sequences on one plasmid can potentially give rise to recombinations or other difficulties during cloning, but we observed no difference in cloning efficiency when using a vector that contained two expression cassettes compared to the normal expression vector. In a similar fashion, no obvious differences were observed upon transfection to *Dictyostelium*; Typically, a few hundred colonies appeared about 3 days after electroporation. Cells that co-express GFP (G418 resistance) and mRFPmars (hygromycin resistance) on different vectors are shown in the left panel of Fig. 5A. It can be seen that there is some cell-to-cell variation in the level of green and red fluorescence. Similar variation has been reported previously for other extrachromosomal vectors (Blaauw et al., 2000; Levi et al., 2000). Interestingly, there seemed to be no correlation between the expression levels of GFP and mRFPmars (Fig. 5B and C, left panels). In contrast, when both fluorophores are expressed on a single vector there is a very high degree of correlation between the expression of GFP and mRFPmars (Fig. 5, right panels). Apparently, two genes that are physically located close to another and are under the control of separate, but identical promoter and terminator, share similar expression levels. The high degree of co-expression of the one plasmid system will be of great benefit for the analysis of co-localization between two proteins.

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